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Identification of Rabbit Myostatin Gene Polymorphisms

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ABSTRACT

The existence of selection on the rabbits with potential for meat has only been seen from phenotypic aspects including performance and productivity, while the molecular genetic studies are still very rare. One of the candidate genes for meat production traits in rabbit is myostatin. Totally 50 blood samples of male rabbits from Rex, Satin, Reza (crossing from Rex and Satin), Flemish Giant and FZ3 (crossing from Flemish Giant and Reza) breed were used at Indonesian Research Institute for Animal Production (IRIAP). Genetic polymorphism by Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) method used *FspBI* restriction enzyme. PCR-RFLP data were analyzed by calculating allele and genotype frequencies. Sequencing was performed in rabbit with different genotypes which represents each of the samples. Genotype of AT had two cut points of the *FspBI* restriction enzyme at the base position of 508 bp and 444 bp. The cut point at the base position of 446 bp was site mutation base T became A. Genotype of TT had one cut point at the base position of 508 bp and no mutation site. Allele T had higher frequency than allele A and just Rex and Reza rabbit breeds had two alleles. The other rabbits (Satin, Flemish Giant and FZ3) only had one allele i.e., allele T. PCR - RFLP analysis of the MSTN|*FspBI* gene segments was polymorphic in Rex and Reza rabbit breeds. All of rabbit breeds in this study did not have AA genotype.

Key words: myostatin gene, rabbit

ABSTRAK

Seleksi pada kelinci pedaging pada umumnya hanya dilihat dari aspek fenotipik termasuk performa dan produktivitas, sedangkan studi pada aspek genetik masih jarang dilakukan. Salah satu kandidat gen yang berhubungan dengan sifat produksi pada kelinci ialah gen myostatin (MSTN). Sampel darah berasal dari 50 kelinci pejantan bangsa Rex, Satin, Reza (silangan Rex dan Satin), Flemish Giant, dan FZ3 (silangan Flemish Giant dan Reza) yang dikoleksi dari Balai Penelitian Ternak Ciawi. Identifikasi keragaman genetik menggunakan teknik PCR-RFLP (*polymerase chain reaction – restriction fragment length polymorphism*) dengan *FspBI* sebagai enzim pemotong serta dilakukan perhitungan frekuensi alel dan frekuensi genotipe. Sekuensing dilakukan pada kelinci dengan genotipe yang berbeda. Genotipe AT memiliki dua titik potong pada posisi 508 pasang basa (pb) dan 444 pb. Titik potong pada posisi basa ke-446 pb merupakan situs mutasi basa T menjadi A. Genotipe TT memiliki satu titik potong pada posisi basa ke 508 pb dan tidak ditemukan situs mutasi. Alel T memiliki frekuensi paling tinggi dibandingkan dengan alel A dan hanya kelinci Rex dan Reza yang memiliki dua alel. Bangsa kelinci yang lainnya, yaitu kelinci Satin, Flemish Giant, dan FZ3 hanya memiliki satu alel, yaitu alel T. Analisis PCR-RFLP pada segmen gen MSTN|*FspBI* ditemukan polimorfik pada kelinci Rex dan Reza. Semua bangsa kelinci pada penelitian ini tidak memiliki genotipe AA.

Kata kunci: gen myostatin, kelinci

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INTRODUCTION

Rabbit is one of the potential animal and can be used for experimental or development for meat production (Lebas *et al.,* 1997; Kazutoshi, 2009; Shuji, 2009). Identification of genes related to the economic trait in rabbit was important for improvement and development of genetic quality. Currently, the existence of selection and crossbreeding performed in rabbits with potential for meat in Indonesia are usually seen from the aspect of phenotype includes performance and productivity, while the genetic aspects (gene) is still limited. Identification of gene in Indonesian rabbit has investigated by Amalianingsih *et al.* (2014). Selection by phenotypic aspect must be done in a longer time with economically expensive. But, the condition will be different if selection is conducted by genetic approach. One of the candidate genes for meat production traits in rabbit is myostatin (Fontanesi *et al.* 2008).

Myostatin as a member of TGF-β (transforming growth factor-β) superfamily was identified as the factor causing double muscling (Bellinge *et al*., 2005) and change of phenotypic. Association of myostatin gene and production trait has been reported in other livestock such as cattle (Sellick *et al*., 2007; Gill *et al.,* 2009; Wiener *et al*., 2009), sheep (Tellam *et al.,* 2012), pig (Stinckens *et al*., 2008), chicken (Zhang *et al.,* 2011), horse (Dall'Olio *et al*., 2014), and rabbit (Bindu *et al*., 2012). Study of myostatin gene in rabbit has been done by Rafayova *et al.* (2009) and Markowska *et al*. (2011).

Rex, Satin, Reza (crosses Rex and Satin), FZ3 (crosses Reza and Flemish Giant), and Flemish Giant are rabbits that are used to develop breed in IRIAP which is a germplasm rabbit meat in Indonesia.

Rex, Satin, and Reza are the rabbits with potential for meat and leather-fur. For information, Rex was one of rabbit clump imported in 1988 from the United States. Rex has been tested in laboratory (Research Institute, and Sub-Research Institute Klepu, Ungaran) and some fields, i.e. Pandansari (Brebes), Wonosobo (Central Java), Makassar (South Sulawesi), Cisarua and Bandung (West Java), but this breed can not be categorized as local breed because the selected phenotypic trait is not much different from the origin clump (Brahmantiyo *et al.,* 2010).

FZ3 and Flemish Giant are the rabbits with potential for meat and have higher body weight than other rabbit breeds (Brahmantiyo, 2008). Identification of gene for these rabbits will be important for improvement of genetic quality for marker-assisted selection.

This research therefore was aimed to analyze the MSTN|*FspBI* gene polymorphism in Rex, Satin, Reza, FZ3, and Flemish Giant rabbit breeds. Identification about polymorphism as basic information and in another time can be associated with production traits.

MATERIALS AND METHODS

Blood Samples

Totally 50 blood samples of rabbit were used that were collected from 5 breeds, consisting of Rex (18 samples), Satin (11 samples), Reza (11 samples), Flemish Giant (4 samples), and FZ3 (6 samples) at Indonesian Research Institute for Animal Production (IRIAP). Blood samples had already been extracted as DNA collections at the Animal Molecular Genetic Laboratory, Faculty of Animal Science, Bogor Agricultural University.

DNA Extraction

Five milliliters of blood samples were collected from each rabbit in non-anticoagulant polypropylene tubes. Blood samples were then mixed with 96% ethanol. The process of DNA isolation used phenol-chloroform method (Sambrook *et al*., 1989). Genomic DNA was stored at -20 °C until amplification with polymerase chain reaction (PCR).

DNA Amplification

Amplification of Polymerase Chain Reaction (PCR) was carried out by using specific primer for parts of the part of intron 1, exon 2, and part of intron 2 (570 bp). Primers used were for forward 5'- TGCATGCATTATCCCAATAGA -3 'and reverse 5'- TCGGTAGTTGTTTCCCACTTT -3' (Fontanesi *et al*., 2011). The PCR was performed in a final volume of 15 μL for each reaction containing 1 μL of DNA sample, 9.35 μL distillated water, 0.3 μL primers, 0.05 μL Taq polymerase, buffer 3 μL, 0.3 μL dNTPs, and 1 μL $MgCl_2$. The reaction mixture was subjected to an initial 5 min of denaturation at 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing for 30 s at 57 °C, extension for 30 s at 72 °C and a final extension for 5 min at 72 °C.

PCR-RFLP Analysis

Genetic polymorphism of the MSTN gene was done by Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) method using *FspBI* restriction enzyme. This enzyme recognized and cut at nucleotides of C|TAG sites. The other method for genotyping of MSTN gene in rabbit was studied by Peng *et al.* (2013).

Visualization of amplification was analyzed on Agarose gel 1.5% containing 2.5 μL EtBr (ethidium bromide) and 0.5X TBE buffer (1 M Tris, 0.9 M Boric acid, 0.01 M EDTA pH 8.0) with a 100 bp ladder as a molecular weight marker for confirmation of the length of PCR product. Digestion by using enzyme and determination of RFLP, 5 μL of PCR products was added to 0.3 μL *FspBI* enzyme, 1 μL distillated water, and 0.7 μL R buffer. The mixture was then incubated at 37 °C for 16 h. The digestion products were separated by horizontal electrophoresis (100 volts, 40 min) in 2% agarose gel in 0.5 X TBE and 2.5 μL ethidium bromide visualized on UV transiluminator.

Sequencing Analysis

Sequencing was performed in rabbit with different genotypes which represents each of the samples.

Sequencing was performed by using a machine sequencer (ABI 3100-Avant Genetic prims Analyzer) in forward and reverse primer fragments. Sequences were analyzed by using MEGA 5 software and BioEdit software.

Data Analysis

PCR-RFLP data were analyzed by calculating allele and genotype frequencies (Nei & Kumar, 2000). Genotype frequency, determined by the calculation of the ratio of a specific genotype in each population, was calculated by the following formula:

$$x_{ii} = n_{ii}/N$$

Allele frequency was calculated as ratio of a certain allele to the overall alleles at a certain locus in a population (Nei & Kumar, 2000). Allele frequency of MSTN gene|FspBI was calculated by the following formula:

$$x_i = (2n_{ii} + \sum n_{ij})/2N$$

where x_{ii} is frequency of genotype AiAi, x_i is frequency of allel Ai, n_{ii} is number of genotype AiAi, and n_{ij} is number of genotype AiAj, and N is total samples.

Information content of allele was calculated by PIC values by using method described by Botstein *et al*. (1980) and Nagy *et al.* (2012).

$$PIC = 1 - \sum_{i=1}^{n} Pi^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

where P_i and P_j stand for frequency of band *i* and band *j*, respectively, in one population; *n* is the number of alleles from a certain locus.

RESULTS AND DISCUSSION

Myostatin (MSTN) Gene Amplification

The result of MSTN gene amplification showed that an amplicon with the length of 570 bp which was located in part of intron 1, exon 2, and part of intron 2. Gene segment amplification products were visualized on 1.5% agarose gel as shown in Figure 1.

The amplification fragment of the MSTN gene was performed by Fontanesi *et al.* (2011) with annealing temperature was 57 °C and this research had the same temperature to get amplicon at 570 bp. Kurkute *et al.* (2011) was performed amplification of MSTN gene in exon 2 and got the amplicon at 570 bp but in different annealing temperature. The amplification of the MSTN gene fragment was carried on GeneAmp® PCR System 9700 (Applied Biosystem) with the success rate of the MSTN gene amplification in this study was 100%.

MSTN|*FspBI* Gene Polymorphism

The PCR-RFLP analysis showed that the MSTN|*FspBI* gene segments were polymorphic. But in this study there were two genotypes identified, namely TT and TA genotypes that were derived from two alleles, namely T and A alleles (Figure 2). Genotyping of the MSTN|*FspBI* showed the results one fragment of 508 bp identified for the TT genotype and two fragments of 508 bp and 444 bp for the TA genotype. Genotype of AA was not found in this study.

Genotype of TT had higher frequency than TA genotype in all of rabbit breeds studied. Allele T had higher frequency than allele A and just Rex and Reza breeds having two alleles. The other rabbits just had one allele i.e., allele T (Table 1). Nei & Kumar (2000) stated that an allele was polymorphic if frequency of that allele was equal or less than 0.99. Mutation on the same point of this gene, mainly for the same breed in other countries

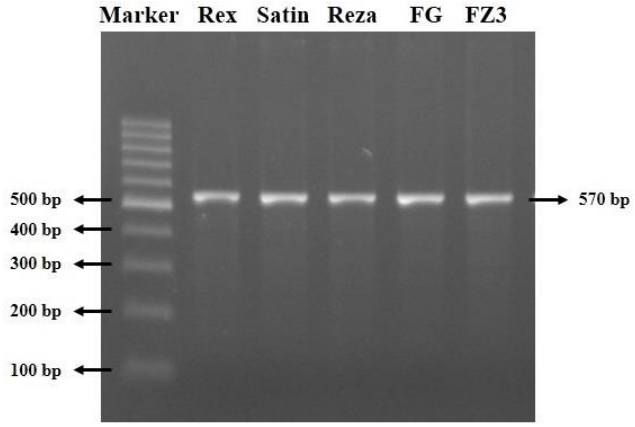

Figure 1. Visualization of MSTN gene amplification results in 1.5% agarose Gel

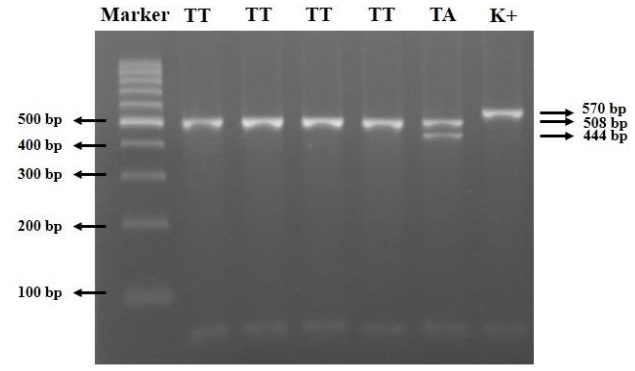

Figure 2. Result of MSTN gene fragment using PCR-RFLP method with FspBI restriction enzyme on 2% agarose gel. Note: TT, TA = Genotype; K+ = PCR product.

Table 1. Genotype and allele frequencies of the MSTN gene

Breed	n	Genotype frequencies			Allele frequencies	
		TT	AA	TA	T	A
Rex	18	0.61	0.00	0.39	0.81	0.19
Satin	11	1.00	0.00	0.00	1.00	0.00
Reza	11	0.64	0.00	0.36	0.82	0.18
FZ3	6	1.00	0.00	0.00	1.00	0.00
Flemish giant	4	1.00	0.00	0.00	1.00	0.00

Note : n = number of sample

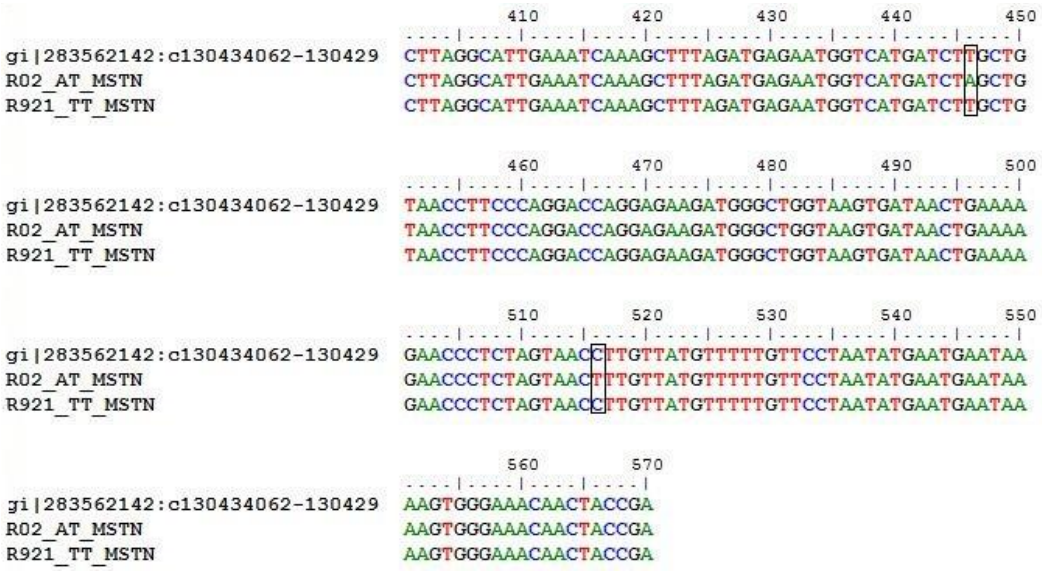

Figure 3. Comparison of gene bank and result of sequencing analysis from different genotype

Table 2. Estimating of polymorphic informative content (PIC) value on rabbit breeds

Breed	n	PIC
Rex	18	0.2604
Satin	11	0
Reza	11	0.2516
FZ3	6	0
Flemish giant	4	0

Note : n = number of sample

has not been found because the genetic research on rabbits are still very rare. Analysis of PIC value was shown in Table 2 and the result indicated that not of all of the rabbit breeds included of PIC were in high category based on Botstein *et al*. (1980). It means that fragment of MSTN|*FspBI* gene has high degree of genetic information in Rex and Reza breeds.

Sequencing Analysis

Sequence analysis for different genotypes using MEGA 4 software and BioEdit software indicated that the amplicon had 570 bp, but for genotype of AT had two cut points of the FspBI restriction enzyme at the base position of 508 bp and 444 bp. The cut point at the base position of 446 bp was site mutation base T become A and at the base position of 516 bp was site mutation base C become T. Genotype of TT had one cut point at the base position of 508 bp and no mutation site as shown in Figure 3.

Two sites of mutations were found in TA genotype at c.446T>A and c.516C>T based of sequencing analyses and gene bank of MSTN gene. Because of visualized by electrophoresis gel is shown of TA genotype of 508 bp and 444 bp, just site mutation at c.446T>A that is looking special. Its because in this study the restriction enzyme has two cut points and caused genotype of TT with no mutation has visualized in agarose gel does not have same base with PCR product.

CONCLUSION

PCR-RFLP analysis of the MSTN|*FspBI* gene segments was polymorphic in Rex and Reza rabbit breeds. All of rabbit breeds in this study did not have AA genotype.

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